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Purification and Properties of GTP:GTP Guanylyltransferase from Encysted Embryos of the Brine Shrimp *Artemia**

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GTP:GTP guanylyltransferase has been purified from the yolk platelets of *Artemia* cysts. The 480-kDa enzyme catalyzes the reversible reaction 2GTP ↔ Gp_4G + PP_i and contains immunologically related polypeptides of 142, 88, and 45 kDa and a distinct 80-kDa component. The 88 and 45 kDa species can be covalently labeled with [α-^{32}P]GTP. Even in crude extracts, the enzyme appears to be partially proteolyzed, suggesting that it is a nonfunctional residue of the pre-encystment stages of development. A native $\alpha_2\beta_2$ structure comprising 2 mol each of the 142- and 80-kDa polypeptides is proposed. The reaction follows ping-pong kinetics with a covalent enzyme-guanylate intermediate containing a phosphoramidate linkage, probably phospholysine. The enzyme has two GTP-binding sites: a "donor" site in which the enzyme-guanylate is formed and which is highly specific for guanine nucleotides (GTP, p_4G, dGTP, and GppNHp) and an "acceptor" site which additionally binds XTP, ITP, GDP, and ADP. Thus, the enzyme will form the homodinucleotides Gp_4G, Gp_5G, Gp_3G, dGp_4dG, and GppNHppG and the heterodinucleotides Gp_4X, Gp_4I, and Gp_3A, but not Xp_4X, Ip_4I, or Gp_4A. The K_m for GTP was 6.7 mM and k_{cat} was 1.6 s^{-1}. XTP was a fully uncompetitive inhibitor of Gp_4G synthesis while ITP was a partially uncompetitive inhibitor. In the reverse reaction, certain pyrophosphate analogs could substitute for PP_i. The structure and mechanism of this enzyme suggest an evolutionary relationship to mRNA capping enzymes.

Adenine-containing dinucleoside polyphosphates, such as diadenosine 5',5'''-P^1,P^4-tetraphosphate (Ap_4A),[1] Ap_3A, Gp_4A, and Gp_3A are found in all cells in concentrations ranging from 0.05 to >100 μM. Their concentrations increase after exposure of cells to certain metabolic stresses (heat, oxidative, nutritional, and DNA damage), and they have therefore been implicated in the regulation of these cellular stress responses (1–3). They are synthesized by the addition of the adenylate moiety of aminoacyladenylates to an acceptor nucleotide (4). The guanine homologs Gp_4G and Gp_3G have also been detected at submicromolar concentrations in *Escherichia coli*, *Saccharomyces cerevisiae* (5), and reovirus transcription mixtures (6, 7) and, in eukaryotes at least, are presumed to be minor products of activities such as mRNA-capping enzymes (8) and Ap_4A phosphorylase (9).

In contrast, Gp_4G and Gp_3G are major nucleotides in the brine shrimp *Artemia* sp. and related Branchiopoda, including *Daphnia magna* (10–12). In *Artemia*, Gp_4G is synthesized in the developing oocyte during vitellogenesis while Gp_3G synthesis occurs in the embryo after fertilization (13). Both compounds are found mainly in the yolk platelets (14). Under certain environmental conditions, embryogenesis in *Artemia* arrests at the 4000 cell stage, and the embryos enter a state of diapause. Ametabolic cysts are formed which can dehydrate and survive for many years. Upon rehydration, development resumes and, 12–16 h later, swimming larvae hatch (15, 16). Gp_4G has been assigned several roles in this process. First, it provides a source of purines for embryonic and larval RNA and DNA synthesis: the rate of *de novo* purine synthesis may be insufficient (10, 11, 17, 18). Second, Gp_4G may serve as a source of phosphate bond energy for maintenance metabolism during anoxic developmental arrest of the cysts (11). Third, it regulates several enzyme activities, including eIF-2(α) kinase (19), GMP reductase (20), and IMP dehydrogenase (21) and so may be important in controlling protein synthesis and adenine nucleotide formation. The concentration of Gp_4G in *Artemia* yolk platelets may be as high as 15 mM while that of Gp_3G, which may also participate in these processes, is about 10-fold lower (10–13).

The enzyme responsible for the synthesis of Gp_4G in *Artemia*, GTP:GTP guanylyltransferase (Gp_4G synthetase), was first isolated from yolk platelets in 1974 (22). It catalyzes the reversible reaction GTP + GTP ↔ Gp_4G + PP_i and so has the potential to mobilize Gp_4G stores. It can also synthesize Gp_3G according to the scheme Gp_4G + GDP → Gp_3G + GTP (23). However, it has proved very difficult to purify due to its insolubility at ionic strengths below 0.2. Thus, few of its properties have been studied in detail. Given the unique occurrence of diguanosine nucleotides in branchiopods, the evolutionary origin and molecular nature of the equally unique enzyme responsible for their synthesis are clearly of interest. Here, we describe the purification of this enzyme to virtual homogeneity. Its properties suggest a structural, mechanistic and evolutionary relationship to a major group of guanylyltransferases, the mRNA-capping enzymes.

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[1] The abbreviations used are: Ap_4A, diadenosine 5',5'''-P^1,P^4-tetraphosphate; Ap_3A, diadenosine 5',5'''-P^1,P^3-triphosphate; Gp_3G, diguanosine 5',5'''-P^1,P^3-triphosphate; Gp_4G, diguanosine 5',5'''-P^1,P^4-tetraphosphate; Gp_5G, diguanosine 5',5'''-P^1,P^5-pentaphosphate; p_4G, guanosine 5'-tetraphosphate; Gp_3A, guanosine 5'-adenosine 5'-P^1,P^3-triphosphate; Gp_4A, guanosine 5'-adenosine 5'-P^1,P^4-tetraphosphate; Gp_4X, guanosine 5'-xanthosine 5'-P^1,P^4-tetraphosphate; Gp_4I, guanosine 5'-inosine 5'-P^1,P^4-tetraphosphate; Gp_4N, guanosine 5'-nucleoside 5'-P^1,P^4-tetraphosphate; dGp_4dG, di(2'-deoxyguanosine) 5',5'''-P^1,P^4-tetraphosphate; Np_3N', dinucleoside 5',5'''-P^1,P^3-triphosphate; Np_4N', dinucleoside 5',5'''-P^1,P^4-tetraphosphate; GppNHp, 5'-guanylylimidodiphosphate; 7-MeGTP, 7-methylguanosine 5'-triphosphate; GTPγS, guanosine 5'-*O*-(3-thiotriphosphate); TLCK, N^{α}-*p*-tosyl-l-lysine chloromethyl ketone; TEA, triethanolamine; IMAC, immobilized metal ion affinity chromatography; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; NMP, nucleoside 5'-monophosphate.

EXPERIMENTAL PROCEDURES

Materials

Artemia cysts were from the Sanders Brine Shrimp Co., Ogden, UT. Calcium tartrate gel was prepared as described (24). TLCK, leupeptin, benzamidine, Reactive Blue 4-agarose, iminodiacetic acid immobilized on Sepharose 6B, butyl-agarose, horseradish peroxidase-conjugated rabbit anti-chicken IgG, and 3,3'-diaminobenzidine were from Sigma. Sephacryl S-400 was from Pharmacia, Sweden. The NeoBar AQ anion-exchange column was from Dynatech, Norway. [8-^{3}H]GTP (7 Ci/mmol), [α-^{32}P]GTP (650 Ci/mmol), [γ-^{32}P]GTP (600 Ci/mmol), and [α-^{32}P]ATP (650 Ci/mmol) were from Amersham. Calf intestine alkaline phosphatase was from Boehringer. The *t*-alkyl amine Primene-JMT was from Rohm and Haas. Gp_4G was prepared from *Artemia* cysts and all other nucleotides were from Sigma or Boehringer. Phosphonate analogs of pyrophosphate were generous gifts from Drs C. E. McKenna, University of Southern California and D. W. Hutchinson, University of Warwick and ephippial eggs of *D. magna* were kindly donated by Dr. A. H. Warner, University of Windsor.

Gp_4G Synthetase Assays

Liquid Ion-exchange Method—The assay, modified from that of Warner and co-workers (22), contained 20 mM TEA-HCl, pH 5.9, 20 mM magnesium acetate, 10 mM 2-mercaptoethanol, 2 mM [^{3}H]GTP (1 mCi/mmol) in a total volume of 125 µl and was incubated at 43 °C for up to 2 h. The pH was then adjusted to 9.5 by the addition of 325 µl of 58 mM Na_2CO_3-$NaHCO_3$ buffer (pH 10.2) containing 5 units of alkaline phosphatase and the excess [^{3}H]GTP converted to [^{3}H]guanosine by incubation at 37 °C for 1.5–2 h. Next, 110 µl of 1.25 M sodium formate buffer (pH 4.0) and 600 µl of 0.1 M Primene-JMT dissolved in amyl acetate were added, the mixture shaken thoroughly three times for 15 s each, then centrifuged at 700 × *g*. The [^{3}H]Gp_4G formed in the assay transferred to the upper amyl acetate layer to form the amine salt, leaving [^{3}H]guanosine in the aqueous layer. The upper layer (450 µl) was removed and the radioactivity determined. One unit of enzyme activity was defined as the amount that synthesizes 1 nmol of Gp_4G/min under standard assay conditions.

HPLC Method—This method was used to determine Gp_4G synthetase activity with different substrates. Reactions containing 20 mM TEA-HCl, pH 5.9, 15 mM magnesium acetate, 10 mM 2-mercaptoethanol and substrate in a volume of 100 µl were incubated at 40 °C for up to 1 h, boiled for 3 min, centrifuged at 16,000 × *g* for 2 min, then applied to a 50 × 7.8 mm Bio-Gel MA7Q column (Bio-Rad) equilibrated in 5 mM ammonium bicarbonate (pH 8.6). Unless otherwise stated, elution was with a 15-min linear gradient from 50 to 570 mM ammonium bicarbonate (pH 8.6), at 1.5 ml/min. Where possible, peaks were identified by comparison with authentic standards and were quantitated by integration.

Purification of Gp_4G Synthetase

All operations were performed at 4 °C unless otherwise stated, and the liquid ion-exchange assay was used to monitor the enzyme activity of column fractions.

Step 1: Preparation of Crude Extract—Cysts (200 g) were hydrated overnight in 2 liters of artificial seawater (0.4 M NaCl, 0.009 M KCl, 0.05 M $MgCl_2$, 0.009 M $CaCl_2$, and 0.028 M Na_2SO_4 (pH 8.0)), decapsulated in sodium hypochlorite at room temperature (25), then filtered. The wet cake was suspended in 1600 ml of cold buffer A (25 mM TEA-HCl (pH 7.5), 5 mM magnesium acetate, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 1 mM benzamidine, and 10 µM $CuCl_2$) plus 1 µg/ml leupeptin, 50 µM TLCK, 0.25 M sucrose, and 0.1% Tween 80, and homogenized with a loose-fitting Potter homogenizer. Crude yolk platelets were washed twice by centrifugation (700 × *g*, for 5 min) with buffer A containing sucrose and Tween as above and extracted in 400 ml of buffer A containing 2 M NaCl at 4 °C for 1 h. The extract was centrifuged at 91,000 × *g* for 1 h, the supernatant dialyzed against buffer B (buffer A containing 0.2 M NaCl) overnight at 4 °C, then centrifuged at 14,000 × *g* for 15 min.

Step 2: Calcium Tartrate Chromatography—Crude extract (590 ml) was applied at 90 ml/h to a 140 × 52-mm column of calcium tartrate gel equilibrated with buffer B. After eluting the unbound protein, a linear gradient of 0–0.4 M potassium phosphate in buffer B was applied. Fractions (18 ml) were assayed for Gp_4G synthetase activity after dialysis of 200-µl samples against buffer B for 3 h to remove inhibitory phosphate. Enzyme activity eluted between 0.14 and 0.28 M potassium phosphate. Active fractions were pooled, giving a volume of 410 ml (Fraction 2).

Step 3: Butyl-Agarose Chromatography—Solid $(NH_4)_2SO_4$ was added to Fraction 2 to a final concentration of 1 M and the sample was loaded at 90 ml/h on to a 110 × 26-mm column of butyl-agarose equilibrated with buffer B containing 1 M $(NH_4)_2SO_4$. After eluting the unbound protein, a reverse linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in buffer B was applied. Dialyzed samples (200 µl) of the 18-ml fractions were assayed. Gp_4G synthetase eluted between 0.65 and 0.3 M $(NH_4)_2SO_4$. Active fractions (260 ml) were concentrated to 20 ml by dialysis against solid sucrose (Fraction 3).

Step 4: Sephacryl S-400 Chromatography—Samples (2 × 10 ml) of Fraction 3 were separately chromatographed at 50 ml/h on a 840 × 28-mm Sephacryl S-400 column equilibrated with buffer B containing 15% glycerol (buffer C). Active fractions (10 ml) from both runs were pooled (Fraction 4).

Step 5: Reactive Blue 4-Agarose Chromatography—The pooled fractions from the Sephacryl column (450 ml) were directly pumped at 100 ml/h through a 100 × 16-mm column of Reactive Blue 4-agarose equilibrated with buffer C. After elution of the unbound protein, activity was removed with buffer C containing 0.6 M NaCl. The eluate (210 ml) was assayed and dialyzed overnight against 25 mM Tris acetate buffer (pH 7.0), containing 0.2 M NaCl (buffer D) to give Fraction 5.

Step 6: Immobilized Metal Ion Affinity Chromatography (IMAC)—Fraction 5 was applied at 100 ml/h to a 170 × 14 mm Zn^{2+}-IMAC column equilibrated with buffer D. This column was prepared by saturation of Chelating-Agarose (Sigma) with Zn^{2+} ions. After the unbound protein had been removed, enzyme activity was eluted with buffer D containing 20 mM histidine, then dialyzed overnight against buffer A containing 50 mM NaCl, 15% glycerol and 10% betaine (buffer E) (Fraction 6).

Step 7: Anion-Exchange Chromatography on Neobar AQ—Samples (4 ml) of Fraction 6 were loaded separately at 3 ml/min at room temperature on to a 4-ml Neobar AQ column equilibrated with buffer E. After eluting the unbound protein, a linear gradient of 50–400 mM NaCl was applied. Fractions (0.5 ml) were assayed and pooled.

A summary of the purification procedure is presented in Table I.

Production and Affinity Purification of Antibodies and Immunoblotting

Fraction 7 from two preparations was subjected to preparative SDS-polyacrylamide gel electrophoresis in a 100 × 100 × 1.5-mm gel at 66 mA and 10 °C for 7 h. The gel was washed briefly in distilled water several times then stained with 0.3 M $CuCl_2$ for 5 min (26). The 88 kDa band was excised, washed with 0.03 M EDTA (4 × 10 min), then with distilled water (4 × 5 min). Next, it was homogenized in 20 ml of PBS containing 1% SDS, then centrifuged at 16,300 × *g* for 15 min. The supernatant was concentrated in an Amicon stirred cell with a 30-kDa cutoff membrane to give 400 µg of the polypeptide in 1 ml.

A polyspecific antibody to Fraction 7 and a monospecific antibody to the 88-kDa polypeptide were prepared as follows. Concentrated Fraction 7 (300 µg in 0.5 ml of TEA-HCl (pH 7.5)) or concentrated 88-kDa polypeptide (200 µg in 0.5 ml of PBS) were emulsified with an equal volume of Freund's complete adjuvant and injected into the pectoral muscle of two hens. A further injection of the same amount of emulsified antigen was performed 16 days later. After the antibody titer of individual eggs reached a maximum, antibodies were prepared from pooled egg yolks (27).

Affinity purification of the antibodies specific for the 88-kDa polypeptide from the polyspecific antibody was carried out according to Smith and Fisher (28). Fraction 7 (12 µg) was applied in a single 7.6-cm wide well to a 7.6 × 6.5-cm 10% SDS gel and electrophoresed at 200 V and 10 °C for 75 min. The separated polypeptides were transferred to a nitrocellulose membrane at 150 mA and 4 °C for 3 h. The blot was blocked in Tween 20 then incubated at 4 °C overnight with a 1:500 dilution of the polyspecific antibody to Fraction 7. After identifying the position of the 88-kDa band by development of a vertical strip cut from the blot, the band of interest was excised horizontally from the original, undeveloped blot and transferred to a microfuge tube. The nitrocellulose fragment was eluted with 3 × 30 s washes of 0.5 ml of 5 mM glycine-HCl (pH 2.3), 500 mM NaCl, 0.5% Tween 20, 100 µg/ml bovine serum albumin. The eluates were immediately neutralized by the addition of Na_3PO_4 to a final concentration of 50 mM.

Immunoblotting was carried out by standard procedures. Blots were incubated at 4 °C overnight in either polyspecific or monospecific chick antibody diluted 1:500. Incubation with second antibody (horseradish peroxidase-conjugated rabbit anti-chicken IgG, diluted 1:5000) was for 5 h at room temperature. Blots were developed with 3,3'-diaminobenzidine and $NiCl_2$ (29).

Antibody-linked Gp_4G Synthetase Polypeptide Assay

The procedure was based on previously reported methods (30, 31). Fraction 7 (0.7 mg) was electrophoresed through three preparative 100 × 100 × 1 mm 10% SDS-polyacrylamide gels which were then stained with $CuCl_2$. The bands of interest were excised, washed with EDTA and distilled water, homogenized in PBS containing 1% SDS, and centrifuged to yield solutions of the various polypeptides. Nitrocellulose membrane squares (1 × 1 cm) were mixed with the various polypeptide solutions (20 μg/ml, 10 ml each) at room temperature for 6 h, blocked with PBS containing 2% Tween for 3 h, and washed (3 × 5 min). The membranes were incubated at room temperature for 3 h with 10 ml of a 1:100 dilution of the anti-Fraction 7 polyspecific antibody in Tween-PBS, washed (6 × 5 min) with 20 mM TEA-HCl buffer (pH 7.5), 200 mM NaCl, 10 mM magnesium acetate, incubated at 4 °C overnight with 5 ml of Fraction 6 enzyme, and washed (6 × 5 min) at 4 °C with the same buffer. Each square was assayed for enzyme activity in 250 μl using the liquid ion-exchange method with 2.5 μCi of [^{3}H]GTP/assay. After completion of the reaction, the mixture was shaken for 1 min, and a 125-μl sample processed as described above.

Active Site Labeling with [α-^{32}P]GTP

Reactions were carried out at 4 °C for 10 min in a final volume of 50 μl containing 20 mM TEA-HCl (pH 6.0), 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 5 μCi of [α-^{32}P]GTP (650 Ci/mmol), and 10 μg of Fraction 7 protein, then terminated with 10 μl of 2 mM GTP and 200 μl of 20% trichloroacetic acid. After 1 h at 4 °C, samples were centrifuged at 16,000 × *g* for 5 min, the supernatants removed, the pellets resuspended in 1 ml of trichloroacetic acid, and centrifuged again. The pellets were resuspended in 1 ml of cold acetone, left at −20 °C for 10 min, and spun. This was repeated three times. The pellets were dried, dissolved in 30 μl of 0.06 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.01% bromphenol blue, 5% 2-mercaptoethanol, and electrophoresed through a 10% SDS-polyacrylamide gel. The gel was then stained in Coomassie Blue, dried on to chromatography paper, and autoradiographed (16 days at −70 °C).

Other Methods

SDS-polyacrylamide gels were run according to Laemmli (32). Protein concentrations were determined by the Bradford method (33).

RESULTS

Purification of the Gp_4G Synthetase—The purification procedure was designed to maintain $I > 0.2$ to prevent precipitation and loss of enzyme activity. The initial extraction buffer contained a mixture of protease inhibitors that has been shown to eliminate the activity of the major *Artemia* cysteine protease (34). Fig. 1 shows the elution profiles of some of the major purification steps. Calcium tartrate chromatography gave a 30-fold purification of the yolk extract and removed most of the "sticky" yolk material (Fig. 1*A*). The properties of this cheap and easily prepared gel are similar to those of hydroxyapatite, which was used in the early purification scheme (22). A variable degree of heterogeneity was observed in the enzyme elution profile; this was not investigated further.

Chromatography on butyl-agarose (Fig. 1*B*) produced only a 2–3-fold purification but improved the efficiency of subsequent steps. The final step, high performance anion-exchange chromatography on Neobar AQ, was carried out at a relatively low ionic strength in order to achieve binding of the enzyme to the column. The inclusion of the zwitterionic betaine at 10% prevented precipitation and also reduced heterogeneity in the elution profile. Fig. 1*C* shows that the enzyme activity of the final fraction (Fraction 7) eluted coincidentally with the major protein peak, suggesting near homogeneity. A final purification of 1700-fold with respect to the crude yolk platelet extract was achieved with a yield of 6% (Table I). The purity of this fraction could not be assessed by native polyacrylamide gel electrophoresis or isoelectric focussing owing to precipitation of the protein in the gel.

Homogeneity of Fraction 7 was also suggested by chromatography of a sample on a calibrated 300 × 7.5 mm Bio-Gel SEC 40XL column. A single, sharp peak of protein of 480 kDa was detected. This value agrees closely with the previously determined figure of 490 kDa (22). However, when analyzed by denaturing electrophoresis in a 10% gel, considerable and variable heterogeneity was seen. All preparations studied (at least 10) showed a minor polypeptide of 142 kDa (*band a*), major bands of 88 (*band b*) and 80 kDa (*band c*), and variable amounts of bands in the range 45–75 kDa (Fig. 2, *lane 1*). Upon extensive dialysis of several independently purified samples of

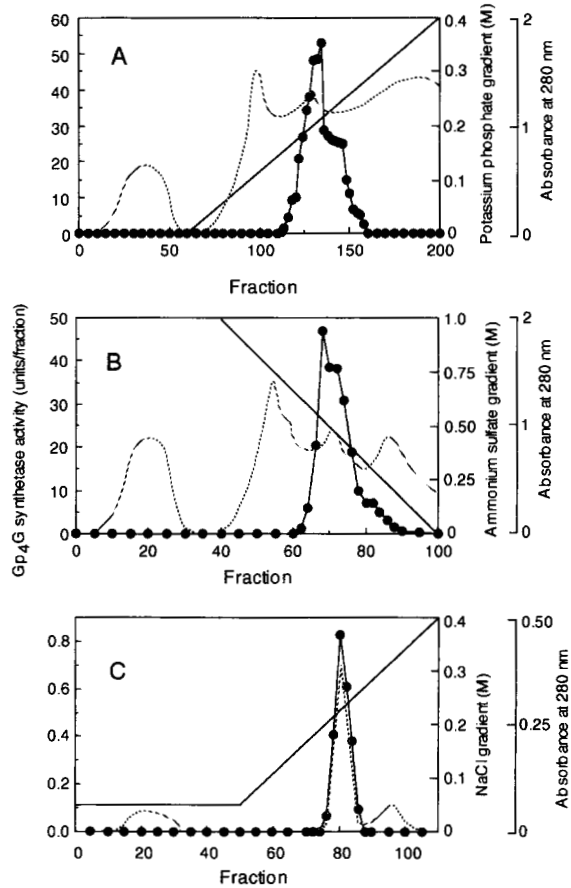

FIG. 1. **Purification of Gp_4G synthetase.** *Panel A*, calcium tartrate chromatography of the crude yolk platelet extract. The sample was applied in 0.2 M NaCl and eluted with a linear gradient of 0–0.4 M potassium phosphate in 0.2 M NaCl as described under "Experimental Procedures" to yield Fraction 2. *Panel B*, butyl-agarose chromatography of Fraction 2. Bound proteins were eluted with a reverse linear gradient of 1–0 M $(NH_4)_2SO_4$ in 0.2 M NaCl as described under "Experimental Procedures" and concentrated to give Fraction 3. *Panel C*, Neobar AQ anion-exchange chromatography of Fraction 6 (Fraction 3 purified further through Sephacryl S-400, Reactive Blue 4-agarose, and by Zn^{2+}-IMAC chromatography). Gp_4G synthetase activity (●), A_{280} (· · · ·), salt gradients (——).

TABLE I

Purification of Gp_4G synthetase from encysted embryos of Artemia

The results shown are for a typical purification. Similar results were obtained with at least 10 purifications.

Step	Volume	Protein	Activity	Specific activity	Purification	Yield
	ml	*mg*	*units*[a]	*units/mg*	*-fold*	*%*
1. Crude extract	590	7964	531	0.067	1	100
2. Calcium tartrate	410	511	964	1.89	28	181
3. Butyl-agarose	260	99	423	4.29	64	80
4. Sephacryl S-400	450	39	452	11.5	172	85
5. Blue agarose	210	18.5	253	13.7	204	48
6. Zn^{2+}-IMAC	175	3.4	162	48.2	720	30
7. Neobar AQ	53	0.30	34	113.0	1687	6.3

[a] One unit of enzyme activity is the amount that synthesizes 1 nmol Gp_4G/min under standard assay conditions.

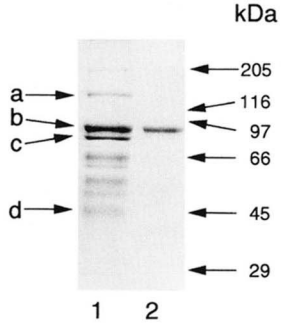

FIG. 2. **SDS-polyacrylamide gel electrophoresis of purified Gp_4G synthetase and isolated 88-kDa band b polypeptide.** Samples were electrophoresed in a 10% SDS gel at 200 V and 10 °C for 1 h and the gel stained with Coomassie Blue. *Lane 1*, 8 μg of Fraction 7 protein; *lane 2*, 2 μg of band b polypeptide isolated from a preparative SDS gel and re-electrophoresed. *Arrows* on the *left* indicate the polypeptides of interest; *arrows* on the *right* indicate the positions of the protein standards (rabbit muscle myosin, *E. coli* β-galactosidase, rabbit muscle phosphorylase *b*, bovine serum albumin, hen ovalbumin, and bovine erythrocyte carbonic anhydrase) and their molecular masses in kDa.

Fraction 7, the 45-kDa band d became more prominent (not shown). These results may reflect the partially proteolyzed status of the enzyme within the cysts.

Polypeptide Composition of the Gp_4G Synthetase—Several procedures were performed to determine if the observed polypeptides were genuine components of the Gp_4G synthetase or contaminants. First, an antibody-linked enzyme assay was performed on the Fraction 7 polypeptides and intervening areas isolated from an SDS gel and immobilized on nitrocellulose (30, 31). The immobilized polypeptides were cross-linked to native Gp_4G synthetase via the two F_{ab} sites of the polyspecific antibody, one of which binds the denatured polypeptides and the other the native enzyme. The nitrocellulose pieces were then assayed directly for enzyme activity using the liquid ion-exchange assay to identify the enzyme polypeptides (having established that the anti-Gp_4G synthetase antibody did not inhibit the activity of the synthetase). Table II shows that bands a–d bound native Gp_4G synthetase activity, suggesting that they are components of the enzyme. Other bands, *e.g.* the 200 kDa band above band a (Fig. 2, *lane 1*) and a negative control, ovalbumin, failed to bind enzyme activity.

As the reaction proceeds via an enzyme-guanylate intermediate (see below), the second method involved covalent labeling of the active site with [α-^{32}P]GTP to identify the catalytic polypeptide. Fig. 3 shows strong labeling of bands b and d. In addition, labeling in the region of band c was evident, although it could not be concluded precisely that this label was associated with band c rather than a degradation product of band b. Band a was only very lightly labeled, the remainder of the label being associated with aggregated protein at the top of the gel. These results suggest that polypeptides b and d (and possibly c) are related in some way as they share a covalent nucleotide-binding site.

To investigate this further, Fraction 7 polypeptides were studied by immunoblotting. An anti-Fraction 7 polyspecific antibody clearly revealed bands a–d in addition to minor polypeptide components, as expected (Fig. 4, *lane 1*). A monospecific antibody to the 88 kDa band b polypeptide was then prepared using band b isolated from a preparative SDS gel as the antigen. Fig. 2 (*lane 2*) shows the purity of the band b antigen used. Interestingly, the anti-band b antibody clearly detected bands a, b, and d, but not band c, when used to probe a blot of Fraction 7 (Fig. 4, *lane 2*). The specificity of detection is shown by the identical pattern obtained in the lane containing crude extract (Fig. 4, *lane 3*). The relationship between bands a and b was also observed using a different monospecific antibody to band b

TABLE II

Antibody-linked enzyme assay of Gp_4G synthetase polypeptides

Individual Fraction 7 polypeptides (200 μg) eluted from an SDS gel were adsorbed on to nitrocellulose squares, incubated with anti-Fraction 7 antibody followed by native Gp_4G synthetase (Fraction 6), washed, then the squares assayed for bound enzyme activity as described under "Experimental Procedures."

Band α[a]	Band b	Band c	Band d	Band above band a[b]	Negative control[c]	Positive control[d]
0.98	1.12	0.99	0.84	0	0	1.1

[a] Figures denote milliunits of Gp_4G synthetase bound/square.
[b] All other regions of the gel examined were negative.
[c] Ovalbumin (200 μg).
[d] Fraction 7 (260 μg) applied directly to a nitrocellulose square after incubation with 2% SDS for several hours at room temperature.

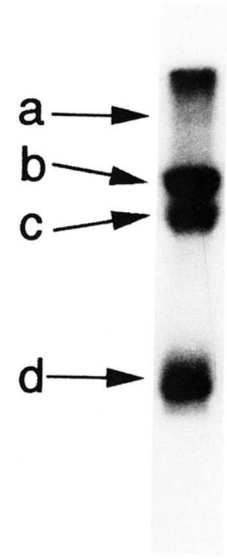

FIG. 3. **Active site labeling of Gp_4G synthetase with [α-^{32}P]GTP.** Reactions were carried out with 10 μg of Fraction 7 and a 10% SDS gel run as described under "Experimental Procedures." The gel was then dried and autoradiographed.

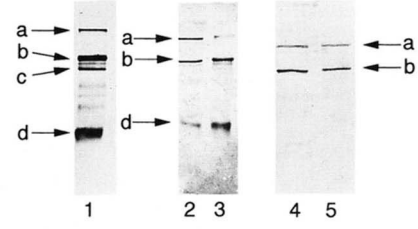

FIG. 4. **Antigenic relationships between Gp_4G synthetase polypeptides.** *Lane 1*, immunoblot of Fraction 7 probed with a polyspecific chick antibody raised against complete Fraction 7; *lanes 2* and *3*, immunoblot of Fraction 7 and crude extract, respectively, probed with a monospecific chick antibody raised against purified band b polypeptide; *lanes 4* and *5*, immunoblot of Fraction 7 and crude extract, respectively, probed with a monospecific antibody to band b polypeptide affinity-purified from the polyspecific anti-Fraction 7 antibody. All methods are described under "Experimental Procedures."

prepared by affinity purification of the band b-specific antibodies from the polyspecific anti-Fraction 7 antibody using band b immobilized on a nitrocellulose membrane (Fig. 4, *lanes 4* and *5*). In this case, cross-reaction with band d was not observed, possibly due to loss of affinity caused by the acidic elution conditions.Together, these results suggest that bands a, b, and d are related and may represent native and partially degraded forms of the catalytic subunit, although why band a was not efficiently labeled by [α-^{32}P]GTP remains to be determined. Band c is probably a distinct polypeptide. Thus, the simplest subunit structure for the 480-kDa enzyme would be $\alpha_2\beta_2$, where

FIG. 5. **Developmental study of Gp_4G synthetase polypeptides.** Immunoblots of crude extracts prepared from developing embryos and larvae of *Artemia* and probed with a polyspecific antibody to Fraction 7 (*A*) and a monospecific antibody to band b prepared by immunizing a chicken with purified band b polypeptide (*B*). *Lanes A–D* are 2 M NaCl extracts of crude yolk platelets (700 × *g* pellet) from 0-, 4-, 8-, and 12-h developing embryos, respectively, and *lanes E–G* are 2 M NaCl extracts of the 700 × *g* pellets from 16-, 20-, and 24-h developing larvae, respectively. *Lanes H–K* are 700 × *g* supernatants (cytosol) from 0-, 4-, 8-, and 12-h developing embryos, respectively, and *lanes L–N* are 700 × *g* supernatants from 16-, 20-, and 24-h developing larvae, respectively.

α is the 142-kDa band a and β is the 80-kDa band c.

Developmental Study of Gp_4G Synthetase Polypeptides—Most (75%) of the Gp_4G synthetase activity of *Artemia* cysts is present in the yolk platelets, with the remainder in the cytosol (14). Since part of the soluble enzyme is of lower molecular weight than the rest and since more of the activity is associated with platelets prepared by non-aqueous procedures, it is possible that the soluble form may represent enzyme undergoing intracellular proteolysis. As this is relevant to the relative roles of the enzyme in Gp_4G synthesis and degradation, a developmental study of both platelet and soluble enzymes was carried out. Cysts were developed in seawater for 0, 4, 8, and 12 h, after which larvae commenced hatching. Larvae were then collected at 16, 20, and 24 h of total development. Crude yolk and cytosolic fractions were prepared from the cysts and larvae, run on a 10% SDS gel, blotted on to nitrocellulose, and the blots probed with both the polyspecific anti-Fraction 7 (Fig. 5*A*) and monospecific anti-band b (Fig. 5*B*) antibodies. Fig. 5*A* shows that between 16 and 20 h, all the enzyme polypeptides disappear from the larvae, concomitantly with yolk platelet degradation (*lanes A-G*). Losses are apparent by 16 h with band c in fact disappearing after 12 h, the time of emergence of the prenauplius larvae. Band d is not prominent in this fraction. In the soluble fraction, band a is virtually absent while band d is clearly evident. Losses are extensive by 12 h and complete by 16 h (*lanes H-N*). Comparable results were obtained using the monospecific anti-band b antibody (Fig. 5*B*). These results suggest that (i) band a is a native platelet-associated polypeptide that gives rise to bands b and d; (ii) band d is produced *in vivo* when the enzyme is released from the platelets or *in vitro* during purification of the platelet enzyme (band b may be generated *in vivo* and *in vitro*); (iii) band c is a distinct polypeptide that is degraded at a rate different from band a; (iv) the bands seen between bands c and d in Fig. 5*A* may be degradation products of band c as they are not detected by the anti-band b antibody (Fig. 5*B*) (they are unlikely to be contaminants as they were detected mainly in the cytosol whereas the Fraction 7 antigen used for antibody production was purified from the yolk platelets).

Are Related Polypeptides Present in Other Organisms?—Immunoblots of extracts from animal (hamster kidney cells, rat hepatoma cells, and human placenta), plant (cabbage, wheat germ, and sweet potato), and fungal (*Aspergillus nidulans*, *S. cerevisiae*) sources were probed with the anti-88 kDa band b antibody. No cross-reaction was observed, while *Artemia* extracts gave a strong positive signal. Surprisingly, even an extract of the ephippial eggs of *Daphnia* contained no cross-reacting material. The 88 kDa band b polypeptide, therefore, appears to be unique to *Artemia*.

TABLE III
Transfer of GMP from enzyme-guanylate intermediate to acceptor nucleotides

The enzyme-guanylate complex was formed using Fraction 6 (0.92 mg) with 2 mM GTP, 20 mM TEA-HCl (pH 6.0), 15 mM Mg acetate, and 10 mM β-mercaptoethanol in a volume of 2 ml. After 1 h on ice, the complex was isolated using a 17 × 9-mm column of Sephadex G-50 equilibrated with 20 mM TEA-HCl (pH 6.0). The enzyme-guanylate complex was recovered in the void volume well separated from free GTP, then concentrated by ultrafiltration under nitrogen pressure and assayed for transfer of the activated guanylyl moiety of the 5′-terminal phosphate of various nucleotides by HPLC as described under "Experimental Procedures" (180 μg enzyme-guanylate complex, 5 mM acceptor nucleotide).

Reaction mixture	Product
Enzyme-GMP without nucleotide	None
Enzyme-GMP + GTP	Gp_4G[a]
Enzyme-GMP + GDP	Gp_3G[a]
Enzyme-GMP + ATP	None
Enzyme-GMP + ADP	Gp_3A[b]

[a] Products were identified based on their elution position relative to the elution position of authentic standards.

[b] This product was tentatively identified on the basis of its elution position.

Reaction Mechanism and Involvement of an Enzyme-Guanylate Intermediate—Many nucleotidyltransferases, including mRNA-capping enzymes, operate via a covalent enzyme-NMP intermediate involving a phosphoramidate linkage between the NMP and a lysine ϵ-NH_2 group on the enzyme (8, 35). The reaction mechanism of Gp_4G synthetase could also involve such an intermediate, or might proceed via direct nucleophilic attack of a non-bridging oxygen of the γ-phosphate of one GTP on the α-phosphorus atom of the second GTP. Labeling of the Gp_4G synthetase polypeptides by [α-^{32}]GTP (see above) strongly supports the former mechanism. To prove that the labeled product is a true intermediate, it was isolated under non-denaturing conditions by gel filtration from a mixture containing enzyme and GTP and shown to catalyze GMP transfer to the acceptor nucleotides GTP, GDP, and ADP, but not ATP, to produce Gp_4G, Gp_3G, and Gp_3A, respectively (Table III). Gp_3G and Gp_3A, but not Gp_4A, have been detected in *Artemia* cysts (36). This is consistent with their being products of the Gp_4G synthetase. The enzyme-guanylate complex is therefore a true reaction intermediate.

The nature of the enzyme-GMP linkage was studied by determining the stability of the ^{32}P-labeled complex in band b isolated from an SDS gel to various chemical treatments (Table IV). By comparison with the chemical sensitivity of the enzyme-AMP phospholysine linkage in T4 DNA ligase, to which it was compared directly (Table IV), and the enzyme-GMP phospholysine linkage in mRNA-capping enzymes (37, 38), the linkage in band b is clearly a phosphoramidate, probably phospholysine.

Properties of the Purified Gp_4G Synthetase—General assay requirements for the enzyme (pH, divalent ion, and temperature optima) were as described previously (22). The K_m for GTP determined from an Eadie-Hofstee plot was 6.7 mM, higher than the previously reported value of 2.2 mM (23), and the V_{max} was 148 units/mg, corresponding to a k_{cat} of 1.6 s^{-1} (assuming homogeneity).

Substrate Specificity—The substrate specificity of the Gp_4G

TABLE IV
Chemical stability of the enzyme-guanylate intermediate

Enzyme-guanylate complexes were formed by incubating 90 μg of Fraction 6, 20 μCi of [α-^{32}P]GTP (650 Ci/mmol), 20 mM TEA-HCl (pH 6.0), 15 mM Mg acetate, 10 mM 2-mercaptoethanol on ice for 1 h. Sample buffer was added to give final concentrations of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.005% bromphenol blue, then the mixture was electrophoresed through a 10% SDS-polyacrylamide gel. Band b, visualized by autoradiography and staining with 0.3 M $CuCl_2$, was cut from the gel, washed with 0.03 M EDTA (4 × 10 min), then washed with distilled water several times. The gel slice was homogenized in 2 ml of 1% SDS and centrifuged at 650 × *g* for 10 min. The supernatant was concentrated with 5 volumes of cold acetone at −20 °C for 10 min and the precipitate resuspended in 75 μl of 0.5% SDS.

Treatment[a]	Band b	T4 DNA ligase[b]
	cpm[c]	
No additions or incubation	1434	6364
0.15 M Tris-HCl (pH 7.5), 5 min, 85 °C	1281	6392
0.15 M HCl, 5 min, 85 °C	137	148
0.15 M NaOH, 5 min, 85 °C	1255	5948
3.86 M hydroxylamine (pH 4.75), 20 min, 37 °C	160	196
0.2 M hydroxylamine (pH 7.5), 20 min, 37 °C	984	6720
4 M sodium acetate (pH 7.5), 20 min, 37 °C	1623	7048

[a] Aliquots (10 μl each) were subjected to various chemical treatments then 2 ml of 5% trichloroacetic acid was added to each sample, the acid-insoluble material collected on glass microfiber filters, and the radioactivity measured.

[b] T4 DNA ligase was used as positive control. The ligase-adenylate complex was formed by incubating 70 μg of DNA ligase (3250 units/mg), 20 μCi of [α-^{32}P]ATP (650 Ci/mmol), 66 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 1 mM ATP on ice for 10 min and was then run on and recovered from an SDS gel as described for the enzyme-guanylate complex.

[c] Trichloroacetic acid-precipitable counts.

synthetase in the forward reaction was studied by HPLC (Table V). In the absence of GTP, only dGTP, GppNHp, and Gp_4 were able to form the corresponding homodinucleotide products, dGp_4dG, GppNHppG, and Gp_5G, while XTP, ITP, dGTP, and GppNHp could form the heterodinucleotide (*e.g.* Gp_4X) in the presence of GTP, in addition to Gp_4G itself. Neither ATP nor 7-MeGTP gave homo- or heterodinucleotide products, suggesting that the carbonyl group on carbon C(6) in G, I, and X and an unsubstituted nitrogen N(7) in G are essential for binding. The lack of any activity with GTPγS suggests that oxygens on the terminal phosphorus are essential for activity while the activity with GppNHp shows that the β-γ bridging oxygen is not essential. Sugar specificity is not absolute as both GTP and dGTP are substrates. These results indicate the presence of two nucleotide-binding pockets with different substrate specificities in the active site of the enzyme: one highly specific for guanine nucleotides and the other less specific and able to accommodate X and I. The question then arises which site has the strict specificity for GTP, the "donor" site, which binds the nucleotide destined to form the enzyme-NMP intermediate, or the "acceptor" site, which binds the NTP receiving the NMP moiety?

This question was resolved by incubating the enzyme with [γ-^{32}P]GTP and XTP as cosubstrates and determining the incorporation of radiolabel into both Gp_4G and Gp_4X by HPLC. If radiolabel appears in Gp_4X, then the donor site is the site of lower specificity; if not, then it is the acceptor site that has the lower specificity. In either case, radiolabel should appear in Gp_4G as the homodinucleotide will always be formed. Fig. 6 shows that only Gp_4G was labeled when using [γ-^{32}P]GTP and XTP as cosubstrates. The [^{32}P]Gp_4G eluted at 17.8 min, 0.6 min after the corresponding peak of UV absorbance due to the dead volume between the UV and radiodetectors; however, there was no peak of radiolabel at 20.6 min, the expected position for [^{32}P]Gp_4X, suggesting that Gp_4G synthetase can form an enzyme-NMP complex only with GTP. The same result was obtained using ITP as cosubstrate. Nucleotidyltransferases such as DNA ligase operate by a ping-pong kinetic mechanism (39). Since GTP is both the first and second substrate for Gp_4G synthetase, we determined the kinetics of inhibition of Gp_4G synthesis by a competing second substrate, XTP. Fig. 7*A* shows that XTP is a fully uncompetitive inhibitor of Gp_4G synthesis. This is consistent with a ping-pong mechanism in which XTP can bind only to the enzyme-guanylate complex. A replot of the *y* intercepts against XTP concentration gave a K_i of 1 mM.

TABLE V
Nucleotide substrate specificity of Gp_4G synthetase

Substrate[a]	Relative synthesis of Np_4N[b]	Relative synthesis of Gp_4N[c]
	%	%
Guanosine 5′-triphosphate (GTP)	100	100
Xanthosine 5′-triphosphate (XTP)	0	22
Inosine 5′-triphosphate (ITP)	0	50
Adenosine 5′-triphosphate (ATP)	0	0
Uridine 5′-triphosphate (UTP)	0	0
Cytidine 5′-triphosphate (CTP)	0	0
Deoxyguanosine 5′-triphosphate (dGTP)	7[d]	96[d]
Guanosine 5′-tetraphosphate (p_4G)	46[e]	50[e]
5′-Guanylylimidodiphosphate (GppNHp)	5[f]	19[f]
7-Methylguanosine 5′-triphosphate (7-MeGTP)	0	0
Guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS)	0	0

[a] All nucleotides were at 2 mM.

[b] The extent of Np_2N synthesis in the absence of GTP relative to Gp_4G synthesis (100%) when only using GTP as substrate.

[c] The extent of Gp_4N synthesis in the presence of GTP relative to Gp_4G synthesis (100%).

[d] The products were tentatively identified as dGp_4dG and dGp_4G on the basis of their elution positions.

[e] The product was identified as Gp_5G by comparison with an authentic Gp_5G standard.

[f] The product was tentatively identified as GppNHppG.

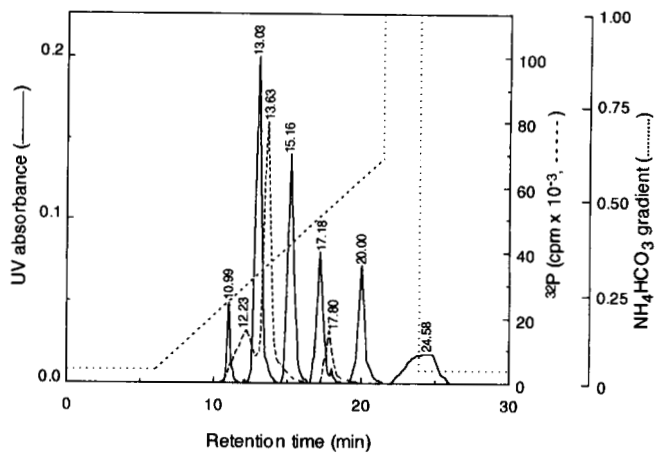

FIG. 6. **HPLC analysis of the reaction products formed from the cosubstrates [γ-^{32}P]GTP and XTP.** Fraction 7 (7 μg) was incubated at 43 °C for 2 h with 2 mM [γ-^{32}P]GTP (8 mCi/mmol), 2 mM XTP, 20 mM TEA-HCl (pH 5.9), 20 mM magnesium acetate, and 10 mM 2-mercaptoethanol in a total volume of 62.5 μl. A 20-μl sample was applied to a Bio-Gel MA7Q column equilibrated in 0.05 M NH_4CO_3 (pH 8.6), using a 15-min gradient from 0.05 to 0.57 M NH_4CO_3. UV absorbance was monitored with a Bio-Rad Dimension™ UV/VIS detector and the radioactivity monitored with a Berthold LB507A continuous flow HPLC radiodetector. The geometry of the HPLC system was such that there was a 0.6-min difference in the retention times of a peak measured by its UV absorbance and by its radioactivity. Peaks were identified as follows: *10.99 min*, GDP (UV); *12.23 min*, PP_i (^{32}P); *13.03 min*, GTP (UV); *13.63 min*, GTP (^{32}P); *15.16 min*, XTP (UV); *17.18 min*, Gp_4G (UV); *17.80 min*, Gp_4G (^{32}P); and *20.00 min*, Gp_4X (UV), respectively. Peak identification was based on known standards except for Gp_4X which was based on its elution position relative to the positions of authentic GTP, XTP, and Gp_4G standards. GDP was present as a contaminant in the GTP substrate used.

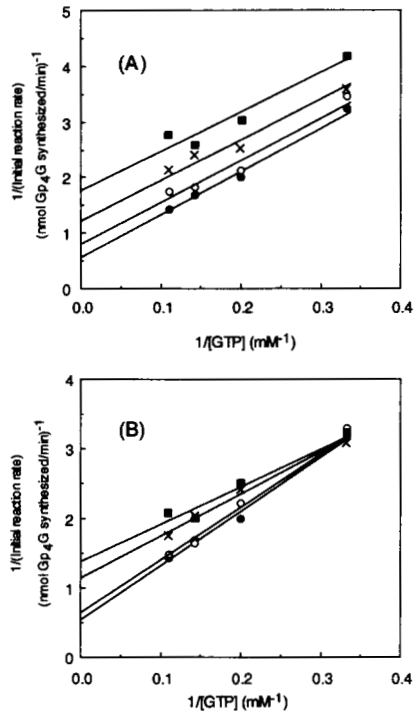

FIG. 7. **Kinetics of inhibition of Gp_4G synthesis by XTP (*A*) and ITP (*B*).** The rate of Gp_4G synthesis was determined by HPLC as described for Fig. 6. Fraction 6 (8 μg) was used. XTP and ITP concentrations were 0 mM (●), 2 mM (○), 4 mM (×), and 6 mM (■).

Curiously, ITP appeared to be a partially uncompetitive inhibitor (Fig. 7*B*) suggesting that this nucleotide is able to promote the formation of the enzyme-guanylate complex at low GTP concentrations. Whether such regulation is of physiological significance during oogenesis remains to be determined.

As the reaction catalyzed by the Gp_4G synthetase is reversible, the ability of pyrophosphate analogs to participate in the back reaction could yield further information on the structure of the active site. With tripolyphosphate ($Na_5P_3O_{10}$), both GTP and Gp_4 were produced; however, with cyclotriphosphate (trimetaphosphate, $Na_3P_3O_9$), only GTP was detected (2 mol/mol Gp_4G) (Table VI). Thus, the strain inherent in the cyclic polyphosphate appears to lead to its hydrolysis under the conditions of the enzyme incubation (40). Of the phosphonate analogs tested, only methylenediphosphonate and carbonyldiphosphonate were substrates. Substitution of one or both methylene hydrogens in methylenediphosphonate with halogens led to loss of activity. Thus, the enzyme can tolerate isosteric substitutions in this position (*e.g.* methylenediphosphonate) better than isopolar substitutions (*e.g.* difluoromethylenediphosphonate).

TABLE VI

Pyrophosphate analog substrate specificity of Gp_4G synthetase

Reactions (125 μl) contained 20 mM TEA-HCl (pH 5.9), 10 mM Mg acetate, 10 mM β-mercaptoethanol, 2 mM PP_i analog, and 0.5 mM GTP and were incubated at 45 °C for 2 h. Fraction 6 enzyme was used and the products analyzed by HPLC as described under "Experimental Procedures."

Substrate	Relative synthesis of GTP analog[a]
	%
Pyrophosphate	100
Tripolyphosphate	75
Cyclotriphosphate	200
Methylenediphosphonate	25
Carbonyldiphosphonate	29
Monochloro-methylenediphosphonate	0
Monobromo-methylenediphosphonate	0
Dibromo-methylenediphosphonate	0
Monofluoromonochloro-methylenediphosphonate	0
Monobromomonochloro-methylenediphosphonate	0
Monofluoro-methylenediphosphonate	0
Difluoro-methylenediphosphonate	0
Monofluoromonobromo-methylenediphosphonate	0
Dichloro-methylenediphosphonate	0
Methylhydroxyl-methylenediphosphonate	0

[a] Extent of synthesis of GTP analog compared with GTP when using pyrophosphate as substrate (100%).

DISCUSSION

The distribution of diguanosine nucleotides in nature is very limited. Within the phylum Crustacea, they have been detected only in the eggs and embryos of the class Branchiopoda (10, 11). They are absent from the somatic cells of these organisms and from all other eukaryotes examined except yeast, where their concentrations are three to four orders of magnitude below those in branchiopods. Thus, in order to understand the evolutionary origin of the mechanism for their synthesis in these crustaceans, a detailed molecular analysis of the enzyme responsible, Gp_4G synthetase, is required. So far, it has proved impossible to prepare the enzyme from *Artemia* cysts, the only readily available source, in a form with a discrete and reproducible pattern of polypeptides. The major protease in these cysts is a cytosolic acid thiol protease, which is completely inhibited by the addition of leupeptin, $CuCl_2$, and TLCK and is inactive above pH 6.5 (34). EDTA and benzamidine were also included to inhibit any minor metalloprotease and serine protease activities that may be present. Despite this, the final product appears to be partially proteolyzed. This conclusion is based upon the immunological relationship between the 142-, 88-, and 45-kDa polypeptides, the apparent presence of a catalytic site on at least the latter two, and the increase in the 45-kDa species upon extensive dialysis or manipulation of the final fraction. Also, release of the enzyme into the cytosol is accompanied by loss of the 142-kDa subunit and the appearance of the 45-kDa species. The species in the 50–75-kDa range may be degradation products of the 80-kDa subunit.

One explanation of these results is that the Gp_4G synthetase is already partially proteolyzed *in vivo* in the cysts: crude extracts display the same heterogeneity in immunoblotting experiments as highly purified fractions, showing that this has not arisen artificially during purification. If the enzyme is only involved in the synthesis of Gp_4G and not in its mobilization, then it will not be required during pre-emergence and subsequent larval development. Hence, it may already be part way along its intracellular degradative pathway, with the first few, specific peptide bonds having been broken prior to encystment. The native 480-kDa structure would still be maintained under non-denaturing conditions. The suggested $\alpha_2\beta_2$ structure, where α = 142 kDa and β = 80 kDa, assumes that band c is unrelated to bands a, b, and d even though there was some evidence of apparent radiolabeling of band c by [α-^{32}P]GTP.

Since the rate of Gp_4G metabolism increases markedly after hatching (10), when the synthetase polypeptides have virtually disappeared, it is unlikely that this enzyme has a role in the mobilization of Gp_4G during early embryonic and larval development. These results, therefore, would not support the proposed model for Gp_4G mobilization in which the platelet enzyme generates Gp_3G from platelet Gp_4G and GDP and the Gp_3G then acts as a phosphate bond energy shuttle, passing into the cytoplasm and being reconverted to Gp_4G by the cytosolic synthetase (10). In this model, the cytosolic Gp_4G is then converted to GMP + GTP by the enzyme Gp_4G pyrophosphohydrolase (EC 3.6.1.17) (41, 42) to provide phosphate bond energy and the purine nucleotides for RNA and DNA synthesis. How-

ever, a role for the synthetase, even in its partly degraded form, in the slow mobilization of Gp_4G during prolonged developmental arrest cannot be excluded.

An alternative mechanism for Gp_4G mobilization has been proposed based on metabolic radiolabeling studies (43). Gp_4G is first converted to Gp_4A by purine base substitution. Gp_4A is then converted to GTP + AMP and GMP + ATP by Gp_4G pyrophosphohydrolase. Interestingly, the intermediate Gp_4A has not been detected in *Artemia* although Gp_3A is present in appreciable amounts along with Gp_3G and Gp_2G (36). If this reaction mechanism involved channeling of enzyme-bound intermediates, the concentration of free Gp_4A would be extremely low, in which case production of free Gp_4A by another mechanism (*i.e.* by Gp_4G synthetase) could affect the progress of this metabolic pathway. This may be the reason why the Gp_4G synthetase has evolved an inability to make Gp_4A.

On the other hand, our results confirm the reported ability of Gp_4G synthetase to make Gp_3G (23) and suggest that it is also responsible for Gp_3A synthesis. In these cases, GMP is donated from the E·GMP to the appropriate nucleoside diphosphate. This mechanism is consistent with that proposed by Warner and Huang (23) for the generation of Gp_3G from Gp_4G and provides a molecular explanation for this reaction, now that the existence of an enzyme-guanylate intermediate has been shown, *i.e.*

$$Gp_4G + GDP \rightarrow Gp_3G + GTP$$

MECHANISM 1

now becomes:

$$E + Gp_4G \rightarrow E\cdot GMP + GTP$$

$$E\cdot GMP + GDP \rightarrow E + Gp_3G$$

MECHANISM 2

The possibility that Gp_2G is also a product of this enzyme with GMP acting as guanylate acceptor was not tested.

As far as the evolutionary origin of the enzyme is concerned, there are a number of interesting similarities between the *Artemia* Gp_4G synthetase and mRNA-capping enzymes. First, both enzymes involve the transfer of a GMP moiety from a phosphoramidate-linked E·GMP intermediate to an acceptor nucleoside polyphosphate to form a 5′-5′ linkage. Second, the specificity of both enzymes for purine nucleotides in the acceptor site is similar. Third, capping enzymes can synthesize Gp_3N and Gp_4N nucleotides from ribonucleoside 5′-di- and triphosphate acceptors. And finally, both capping enzymes and Gp_4G synthetase yield similarly sized intermediates upon proteolysis. Cellular capping enzymes fall into two classes (8): first, there is the "mammalian" type, exemplified by the *Artemia*-capping enzyme. This enzyme comprises a single subunit, separable by proteolysis into two domains containing the guanylyltransferase (44 kDa) and mRNA triphosphatase (20 kDa) activities (44). Second, there is the "yeast" type which has two subunits: a 52-kDa guanylyltransferase polypeptide, which is readily proteolyzed during isolation to a 45-kDa species, and an 80-kDa mRNA triphosphatase polypeptide, which is readily converted to a 39-kDa product (45). These sizes are similar to those observed during isolation of the Gp_4G synthetase, the GTP-binding 88 kDa band b species gives rise to the 45 kDa band d fragment, while band c is 80 kDa and appears also to generate smaller products. Thus, *Artemia* may possess both types of capping enzyme: the mammalian type being used for mRNA capping while the yeast type has evolved into a highly efficient Gp_4G synthetase. Confirmation of this interesting evolutionary possibility, which has ramifications outside *Artemia* biology, and verification of its structure will require sequencing of the Gp_4G synthetase polypeptides and this is now in progress. Both polypeptides of the yeast mRNA capping enzyme have recently been sequenced (46).

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